Contrasting responses of photosynthesis and carbon metabolism to low temperatures in tall fescue and clovers

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Growth, photosynthesis and carbohydrate metabolism in plants of two grassland species, clover (*Trifolium subterra neum* L. cv. Areces and Gaitan) and tall fescue (*Festuca arundinacea* Schreb.), shifted from 25 to 12°C for 1 day or developed at 12°C were compared with controls kept at 25°C. Cold development produced a larger inhibition of growth in fescue than in clovers. In contrast, transferring plants from high to low temperature inhibited photosynthesis to a lesser extent in fescue than in clovers, this difference being associated with an increase in the activation state of Calvin cycle enzymes in fescue, but not in the clovers, a decreased cytosolic fructose-1,6-bisphosphatase (cFBPase, EC 3.1.3.11) activity in clovers, and an accumulation of hexose phosphates only in fescue. Development at 12°C partly relieved the inhibition of photosynthesis in clovers, in contrast with fescue, which correlated with increases in total ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco, EC 4.1.1.39) activity only in clovers, and with greater increases in total stromal FBPase (sFBPase) activity in clovers than in fescue. The activity of sucrose synthesis enzymes was increased in the two clovers and fescue developed in the cold, while carbohydrate accumulation was much bigger in cold-developed fescue than in clovers because of a 5-fold increase in fructan contents in the former. The contents of phosphorylated intermediates increased in clovers but decreased in fescue grown at 12°C. Our results suggest that restricted ribulose-1,5-bisphosphate (RuBP) regeneration limited the recovery of photosynthetic capacity in cold-developed fescue.

Abbreviations – A, Areces; cFBPase, sFBPase, cytosolic and stromal fructose-1,6-bisphosphatase, respectively; F, fescue; FBP, fructose-1,6-bisphosphate; Fru6P, fructose-6-phosphate; G, Gaitan; Glc6P, glucose-6-phosphate; HLT, transfer from 25/15

to 12/8°C (day/night); HT, 25/15°C; LAR, leaf area ratio; LT, 12/8°C; NAa, NAb , net assimilation rate based on real time and thermal time, respectively; PGA, 3-phosphoglycerate; RGRa, RGRb, relative growth rate based on real time and thermal time, respectively; Rubisco, ribulose-1,5-bisphosphate carboxylase oxygenase; RuBP, ribulose-1,5-bisphosphate; SPS, sucrose phosphate synthase; TP, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.

Introduction

At low temperatures, growth and carbon export from leaves often decline more than photosynthesis (Paul et al. 1990, 1992, Martindale and Leegood 1997), resulting in the accumulation of carbohydrate, which may inhibit sucrose synthesis (Hurry et al. 1995b). In the short term, sucrose synthesis is also selectively restricted at low temperature because of the high sensitivity of sucrose phosphate synthase (SPS, EC 2.4.1.14) to temperature and to the decreased affinity of cFBPase for fructose-l,6-bisphosphate (FBP) (Stitt and Grosse 1988), although these kinetic limitations can be compensated by the increase in metabolite pools that occurs at low temperature (Stitt and Grosse 1988, Labate et al. 1990, Hurry et al. 1995b). The inhibition of sucrose synthesis leads to a restriction in phosphate recycling from the cytosol to chloroplasts, which reduces the rate of photosynthetic carbon assimilation (Sharkey et al. 1986, Plaut et al. 1987, Labate and Leegood 1988), through restrictions on photophosphorylation (Labate et al. 1990) or on the carboxylation or regeneration of ribulose-1,5-bisphosphate (RuBP) (Labate et al. 1990, Morcuende et al. 1996). The photosynthetic capacity increases after a period at low temperature (Berry and Björkman 1980) and this is supported by an enhanced activity of the enzymes involved in CO2 fixation, particularly ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) and stromal fructose-1,6-bisphosphatase (sFBPase), and of sucrose synthesis, such as sucrose phosphate synthase (SPS) and cystolic fructose-1,6- bisphosphatase (cFBPase), and by an increase in the pools of phosphorylated intermediates (Holaday et al. 1992, Hurry et al. 1995a,b, Strand et al. 1997, 1999). In contrast, leaves that developed at low temperature did not show an up-regulation of the starch synthesis pathway (Strand et al. 1997, 1999). In addition to sucrose, fructan synthesis is stimulated in grasses at low temperature (Pollock and Cairns 1991) and a higher capacity for CO2 assimilation upon cold acclimation of

winter wheat has been associated with an increased sucrose and fructan biosynthesis (Savitch et al. 2000). In contrast, at 18°C we found a negative relation between fructan contents and rates of photosynthesis (Martı´nez-Carrasco et al. 1993). Soluble sugars (Koster and Lynch 1992) including fructans (Tognetti et al. 1990) and fructan hydrolysis products (Olien and Clark 1993) may exert a cryoprotective function in cells exposed to low temperature, although it has been claimed that there is no absolute requirement for sugars to accumulate in order for tissues to harden (Pollock et al. 1999).

Species and cultivar differences in photosynthetic performance at low temperature have been linked to the ability of plants to activate or to increase the total activity of the enzymes involved in sucrose synthesis and CO2 fixation (Holaday et al. 1992, Hurry et al. 1995a). Differential responses to low temperatures may be particularly relevant for mixed swards, whose biomass and botanical composition may be affected by growth at low temperatures of the various plant species (Collins et al. 1991). However, comparisons of photosynthesis and carbon metabolism at low temperature among these species are scarce. The aim of this study was to investigate low-temperature acclimation in respect of recovery of photosynthesis and activities of enzymes of the Calvin cycle and sucrose synthesis in two contrasting grassland species often grown in mixed swards, clover, which accumulates starch, and fescue, which accumulates fructan. Clover and fescue cultivars recommended for growth under the cold winter, semi-arid environment of the *dehesa* grasslands of Spain were selected. We have examined whether a differential increase in photosynthetic capacity following cold acclimation is associated with a species-dependent stimulation of fructan synthesis. Changes in the activation and total activity of various enzymes after 1 day and several weeks of development at low temperature were assessed along with the amounts of phosphorylated intermediates and carbohydrates. To compare plants grown at different temperatures, the exposure of the

plants to this environmental variable was quantified by summing degree days (Griffith and McIntyre 1993), and analysing plant growth in terms of both thermal time and real time.

Materials and methods

Plant material

Seeds from two clover cultivars (*Trifolium subterraneum* L. cv. Areces and *T subterraneum* L. *brachycalycinum* cv. Gaitan) were germinated on moistened filter paper in a chamber at 25/15°C day/night temperatures and 87% relative relative humidity under low light intensity provided by fluorescent lights in a 16-h photoperiod. Six-day-old clover seedlings and seeds from tall fescue (*Festuca arundinacea* Schreb. cv. Tima) were sown in 6-1 pots (30 plants per pot) containing perlite and placed in a growth room with 70% relative humidity, 300 μmol m^{-2} s^{-1} photon flux density (fluorescent and incandescent lamps) with a 16-h photoperiod, and 25/15°C day/night temperatures (HT). The pots were supplied with water and a nutrient solution (Israel et al. 1990) supplemented with micronutrients (Hewitt 1966). Ten days after planting, when two leaves had expanded, half the pots were transferred to a growth room with 12/8°C (LT). Another group of pots was transferred from high to low temperature (HLT) when the seventh leaf of clovers and the sixth leaf of fescue had fully expanded (25–28-day-old plants). Three replicates from each cultivar×temperature combination, each consisting of 6 pots, were used.

Growth analysis

Samples consisting of 8 plants per experimental variate were harvested every 3 and 6 days in HT and LT plants, respectively. Fresh weight was recorded, leaf area measured with an electronic planimeter (Li-3000 A; Li-Cor, Inc., Lincoln, NE, USA) and, after drying at 80°C for 16 h, dry weight was recorded. Second-degree polynomial regressions of weights and areas over time were fitted with the SIMFIT (W. G. Bardsley, University of Manchester) computer programme. Derived functions [relative

growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR)] were deduced from these growth curves and mean RGR, NAR and LAR over a period of time were obtained by integrating each of the functions with respect to time and dividing by the time interval, according to Radford (1979) and Hunt (1982). One way to analyse the effect on growth of an environmental variable is to quantify exposure to the variable itself by accumulating units over time. Thermal time, which is similar to accumulated temperature, was calculated as the sum over a period of time of the ambient temperature above a threshold temperature of 0°C, following the procedure of Griffith and McIntyre (1993). Growth was analysed over the period from 18 to 28 days for HT plants and from 26 to 46 days for LT plants; this period corresponded to the interval between 400 and 600°C days for both clover cultivars and fescue at both temperatures.

Measurement of photosynthesis

Photosynthetic oxygen evolution at 5% CO2, 900 μmol m^{-2} s^{-1} photon flux density and the growth temperature (25 or 12°C) was measured with a leaf disc electrode (LD2; Hansatech, Kings Lynn, Norfolk, UK) as described by Walker (1987). Measurement was on the seventh leaf of clovers and the sixth leaf of fescue on 1 day after transferring plants from high to low temperature in HT and HLT plants and at the same growth stage (41-44-day-old plants) in LT plants.

Metabolites and carbohydrates

Other leaves (sixth and seventh leaves of fescue and clovers, respectively) were sampled under the conditions of the growth rooms on the same dates as the photosynthesis measurements and immediately transferred to liquid nitrogen under illumination (Krapp et al. 1991, Morcuende et al. 1996). Metabolites were extracted in 1

M $HClO_4$ as described by Labate et al. (1990). The amounts of glucose-6-phosphate (Glc6P), fructose-6-phosphate (Fru6P), FBP, 3-phosphoglycerate (PGA), TP (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate) and RuBP were determined enzymatically according to Kobza and Edwards (1987). Starch in the residue from the $HClO_4$ extractions was digested with amyloglucosidase and amylase and determined according to ap Rees et al. (1977). For measurements of glucose, fructose and sucrose, frozen leaves were extracted with 4.2 ml of extraction medium [1.2 ml $CHCl_3$, 2.4 ml CH_3OH, 0.6 ml buffer containing 50 mM NaF, 10 mM EGTA and 50 mM HEPES (pH 8.5)] as described by Labate et al. (1990) and analysed according to Jones et al. (1977). An estimation of low molecular weight fructans was obtained from the fructose in excess of glucose after invertase hydrolysis. As fructan polymerization does not decline (Pollock 1986), but rather has been found to increase in fescue (Labhart et al. 1983) exposed to low temperatures, our data may underestimate fructan contents at 12°C. The recovery of metabolites added to the extracts in amounts comparable with those present in the tissues was greater than 87%. Chlorophyll (phaeophytin) in the perchloric acid extracts was analysed by the method of Vernon (1960) and that present in the chloroform extracts by the method of Arnon (1949).

Enzyme activities

Subsamples of the stored leaves were ground in a mortar with liquid nitrogen and extracted in buffer for enzyme assays. An aliquot of the whole extract was used to determine chlorophyll contents (Arnon 1949) and the remainder was centrifuged at 13000 *g*. The total time from extraction to the assay of initial enzyme activity was less than 2.5 mm. sFBPase was extracted in the buffer described by Harbinson and Foyer (1991) to which 1% BSA (w/v) and 2% PVP (w/v) were added. cFBPase was extracted

as in Holaday et al. (1992), except that 2-mercaptoethanol was replaced by 1 mM DTT and 1% BSA (w/v) was added. Rubisco was extracted following the procedure of Ward and Keys (1989). FBPase and Rubisco activities were measured in a spectrophotometer at 340 nm as described by Holaday et al. (1992) and Ward and Keys (1989), respectively. SPS was extracted and assayed as described by Huber et al. (1989). Checks were made for the linearity of enzyme activities over time and for the proportionality between rate and amount of extracts.

Statistical analysis

Analyses of variance were performed as in a fully randomized factorial experiment and orthogonal contrasts betweentreatments were made according to Snedecor and Cochran (1967).

Results

Plant growth

Development at 12°C reduced the increase with time in dry mass and green area (Fig. 1A,C) relative to controls at 25°C in the two clovers and fescue. On a thermal time basis (Fig. 1B,D), however, this difference was reversed, the plants accumulating dry mass and green area at a faster rate at low than at high temperatures. Growth analysis based on both real time and thermal time was made over the period from 400 to 600°C days in the two clovers and fescue at both temperatures (18-28 and 26-46 days in HT and LT plants, respectively). Low temperatures increased mean dry weights and green areas over this interval relative to high temperatures (Table 1). In addition, cold development decreased the LAR in the 3 cultivars, and the NAR on a real time basis (NAR_a) in fescue, but not in the clovers. Consequently, low temperature decreased more the RGR on a real time basis (RGR_a) in fescue than in clovers, the decrease being not significant in Gaitan clover. In contrast, cold development increased the RGR on a thermal time basis (RGR_b) in Areces and Gaitan clovers because the increase in thermal time-based NAR (NAR_b) at low temperature was greater than the decrease in LAR. Lowering the temperature had no effect on RGR_b in fescue because the increase in NARb, which was smaller than in clovers, was offset by the decrease in LAR.

Photosynthesis

Transferring plants from 25 to 12°C decreased photosynthetic oxygen evolution at high light and saturating CO_2 (Fig. 2) to a greater extent in clovers, especially in Areces, than in fescue, which therefore showed a higher photosynthetic capacity after a shift to low temperatures. Relative to plants grown at 25°C, development at 12°C decreased photosynthesis less than transfer from 25 to 12°C in clovers, but decreased it to a

similar extent in fescue, indicating that acclimation to low temperatures increased the photosynthetic capacity of clovers.

Carbohydrate content of leaves

A shift from 25 to 12°C increased the contents of glucose and fructose in both clover cultivars (Fig. 3). It also increased sucrose contents in Gaitan clover and fescue, but not in Areces clover, in association with the significant decrease in cFBPase activity in this cultivar (see below). Starch content increased relatively less than soluble sugars in Gaitan clover, but was decreased in Areces clover. Relative to warm controls, cold-developed leaves also contained higher hexose pools in both clover cultivars and higher sucrose pools in Gaitan clover and fescue, the two cultivars with increased cFBPase activity (see below). Starch content was higher in cold- than in warm-developed leaves in Gaitanclover. Notably, the estimated fructan content increased over 5-fold in 12°C fescue leaves, which led to an increase in carbohydrate content markedly higher than in clovers. Thus, in cold-shifted and cold-developed leaves there was an increase in the soluble sugars to starch ratio relative to plants grown at 25°C (data not shown). In addition, in fescue leaves developed at 12°C there was a shift towards increased carbon partitioning into fructan.

Enzyme activities

Lowering the temperature from 25 to 12°C increased the initial activity of Rubisco and sFBPase (Table 2) in fescue by increasing the activation state of these enzymes. In contrast, these initial enzyme activities did not increase in the clovers; rather, total sFBPase activity was significantly decreased in Areces. Development at 12°C increased initial Rubisco and sFBPase activities of clovers over those of warm controls, mainly as a consequence of greater total activities of these enzymes. However, cold development

did not increase total Rubisco activity in leaves of fescue relative, to control leaves at 25°C, and increased total sFBPase activity (1.4-fold as compared with 25°C leaves) less than in clovers (2–3-fold). A temperature shift from 25 to 12°C had no effect on cFBPase activity (Table 2) in fescue, but decreased it in the clovers – significantly in Areces. Cold stress increased maximum SPS activity (Table 2) in the two clovers and fescue, and SPS activation in clovers. Development at low temperatures had no effect on cFBPase activity in Areces clover and increased this activity in Gaitan clover and fescue relative to 25°C controls. Limiting activity of SPS was also greatly increased in the two clovers and fescue relative to warmgrown plants; both the maximum activity and, to a lesser extent, the activation state of the enzyme were increased. Thus, cold acclimation increased the capacity for sucrose synthesis in the two clovers and fescue.

Phosphorylated intermediates

The effects of a decrease in temperature from 25 to 12°C on the contents of phosphorylated intermediates depended on the plant cultivar. The sum of analysed phosphate esters (Fig. 4) did not change in Areces and Gaitan clovers, but increased in fescue. Leaves of Areces clover shifted to 12°C showed reduced PGA, RuBP and TP, but slightly increased Glc6P contents compared with the control leaves. In Gaitan clover transferred to low temperature, TP increased and the rem aining phosphorylated intermediates did not vary significantly. The increase in phosphate esters in fescue plants shifted to 12°C was a result of a marked increase in Glc6P and Fru6P contents, a moderate increase in TP, and decreases in PGA and RuBP relative to controls. Thus, a shift to low temperature increased the TP/PGA and TP/RuBP ratios in Gaitan clover and fescue, indicating, respectively, that the decrease in temperature did not limit the assimilatory force – the combined redox and phosphorylation potential (Dietz and

Heber 1986) – and that RuBP regeneration had become restricted. Transfer to low temperature also increased the Glc6P/Fru6P ratio in clovers, but decreased it in fescue, indicating, respectively, a shift towards slightly higher and lower cytosolic compartmentation of hexose-P (Gerhardt et al. 1987).

Development at 12°C increased the sum of phosphorylated intermediates in leaves of Areces and Gaitan clovers relative to 25°C plants (Fig. 4). In contrast, it decreased the total pool of these intermediates in leaves of fescue. Colddeveloped leaves contained larger pools of PGA and Glc6P in Areces clover, and larger pools of PGA, TP, RuBP, Glc6P, Fru6P and particularly FBP in Gaitan clover relative to control leaves. In contrast, cold development resulted in lower PGA, RuBP, Glc6P, Fru6P and markedly lower FBP in leaves of fescue when compared with plants grown at 25°C; at variance with these intermediates, the pool of TP tended to be higher at low temperature in this cultivar. Consequently, the TP/PGA and TP/RuBP ratios increased in fescue but did not change in clovers developed at 12°C, suggesting that the restriction in RuBP regeneration found in plants of Gaitan clover and fescue shifted from 25 to 12°C did not occur in clovers, but did persist in fescue leaves developed at low temperatures. As in plants transferred from 25 to 12°C, the Glc6P/Fru6P ratio in clovers was higher in cold-developed leaves relative to controls, suggesting an increased cytosolic compartmentation of hexose-P. In contrast, the Glc6P/Fru6P ratio in fescue was similar at 12 and 25°C.

Discussion

Relative to warm controls, cold development produced a larger inhibition of growth in fescue than in clovers, as a consequence of greater reductions in both LAR and NAR in the former species. This differential decrease in LAR at low temperature was associated with higher increases of mean dry weight relative to controls in fescue than in clovers and could be due, at least in part, to greater use of assimilates for the synthesis of compounds with a cryoprotective function rather than for growth (Griffith and McIntyre 1993). Decreased NAR at low temperature was primarily a consequence of the smaller thermal imput, since on a thermal time basis the rate of net assimilation did not decrease. Significantly, cold-developed plants displayed higher thermal time-based NAR relative to warm controls, which suggests an acclimatory stimulation of the capacity for net carbon assimilation. This stimulation was smaller in fescue than in clovers, in agreement with the contrasting response of photosynthesis to cold development in these plants.

Our results show that transfer to low temperature decreased photosynthesis relatively less in fescue than in clovers, and less in Gaitan than in Areces clover. In contrast, clovers displayed an acclimatory enhancement of photosynthesis with development at low temperature that was not observed in fescue. Three factors may have contributed to this differing response to low temperature. First, the transfer to low temperature was correlated with increases in the Rubisco and sFBPase activation state in fescue, in agreement with earlier reports (Holaday et al. 1992 and references therein, Hurry et al. 1994, 1995a,b), but enzyme activation was not increased in clovers. Moreover, sFBPase activity decreased in Areces clover, the most cold-sensitive cultivar, with respect to photosynthesis. Chilling-sensitive tomato cultivars also showed relatively greater losses of sFBPase activity than tolerant ones (Bru¨ggermann et al. 1994). In turn, the partial

recovery of photosynthesis in cold-developed clovers, but not fescue, was associated with increases in total Rubisco activity relative to plants developed at 25°C in clovers but not in fescue, and with higher increases in total sFBPase activity in clovers than in fescue. These results show a differential capacity of clovers and fescue to increase the activity of key enzymes of the photosynthetic carbon reduction cycle, an increase which is required (Holaday et al. 1992, Hurry et al. 1994, 1995b, Savitch et al. 1997, Strand et al. 1999) for recovery of photosynthesis at low temperature.

Second, it has been shown that at low temperatures photosynthesis can be limited by the rate of sucrose synthesis (Labate and Leegood 1988, Stitt and Grosse 1988). In our experiments, total SPS activity increased in both clover cultivars and fescue 1 day after lowering the temperature, in contrast to the slower response of spinach (Holaday et al. 1992) and the increase in activation, but not in total activity, observed in cold-stressed winter rye and wheats (Hurry et al. 1994, Savitch et al. 1997). However, the decrease in cFBPase activity in cold-shifted clovers, especially in Areces, could limit the capacity for sucrose synthesis and was correlated with greater inhibition of photosynthesis as compared with fescue. Development at 12°C restored cFPBase activity to control values in Areces clover, increased cFBPase activity in Gaitan clover and fescue, and led to further enhancements of SPS activity in both clover cultivars and fescue, as observed in other studies (Hurry et al. 1994, 1995a,b, Martindale and Leegood 199, Savitch et al. 1997, Strand et al. 1999). However, this up-regulation of sucrose synthesis capacity, which could counteract the phosphate limitation of photosynthesis at low temperature (Sharkey et al. 1986, Plaut et al. 1987, Labate and Leegood 1988, Strand et al. 1999), was not associated with a recovery of photosynthetic capacity in cold-developed fescue, in contrast with clovers.

Third, an accumulation of phosphorylated intermediates, which has been observed after exposing plants of several species to low temperature for varying periods of time (Labate et al. 1990, Hurry et al. 1994, 1995a,b, Morcuende et al. 1996, Strand et al. 1997), can partially compensate for product inhibition and declining activity of the enzymes at low temperature (Stitt and Grosse 1988, Labate et al. 1990). In agreement with this, the marked increase in hexose-P pools in fescue leaves shifted to low temperature was associated with maintenance of relatively high rates of photosynthesis, in contrast with clovers. Likewise, the increased metabolite contents in cold-developed clovers relative to warm controls could contribute to the partial recovery of photosynthesis in this species. Conversely, lowered metabolite pools, including PGA and RuBP, in 12°C fescue, can restrict RuBP regeneration (Stitt and Grosse 1988) as indicated by the increased TP/RuBP ratio, and limit photosynthesis. Recovery of photosynthesis at low temperature in winter rye (Hurry et al. 1995a,b) and Arabidopsis (Strand et al. 1997) was associated, at least in part, with improvement in the regeneration phase of the Calvin cycle. It may be that decreased metabolite contents and RuBP regeneration are associated with increased fructan synthesis in cold-developed fescue. A similar effect of high rates of sucrose synthesis at low light has been reported (Stitt and Grosse 1988) and we have previously found an association between decreased photosynthesis and fructan accumulation in wheat (Martínez-Carrasco et al. 1993). The reasons for the difference between our results and the positive correlation between CO2 assimilation rate and leaf fructan biosynthesis in cold-acclimated winter wheat (Savitch et al. 2000) are not clear. Despite the possible limitation to the increase of photosynthetic capacity at low temperature infescue, fructan synthesis provides a reserve of carbohydrate, which can be mobilized to sustain faster growth rates when temperature is raised, thus, conferring an adaptive advantage.

Acknowledgements – Dionisio González and Angel Verdejo provided skillful technical assistance. This work was funded by the Junta de Castilla y León.

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Table 1. Mean dry weight and green area and growth parameters over the period from 18 to 27-28 days (25°C, HT) and 26-27 to 45-46 days (12°C, LT) from transplanting, corresponding to the interval between 400 and 600°C days, for clovers and fescue grown at 25 (HT) or 12°C (LT). Data are means of 3 replicates, each consisting of 8 plants. a and b refer to parameters estimated on a real time and a thermal time basis, respectively. LSD, least significant differences (P<0.05) for temperature contrasts. Significant differences are marked with an asterisk.

	Areces		Gaitan		Fescue		LSD
	HT	LT	HT	LT	HT	LT	
Mean dry wt (mg plant^{-1})	92	148*	85	177*	118	272*	50
Green area (cm^2 plant^{-1})	23	33*	18	29*	16	25*	4
LAR (cm^2 mg^{-1})	0.25	0.23*	0.21	0.16*	0.13	0.09*	0.01
NAR_a (mg cm^{-2} d^{-1})	0.50	0.39	0.44	0.45	1.31	0.87*	0.19
RGR_a (mg g^{-1} d^{-1})	124	88*	90	72	164	80*	22
NAR_b (mg dm^{-2} (^{o}C d)$^{-1}$)	2.3	3.6*	2.0	4.2*	6.0	8.2*	0.7
RGR_b (mg g^{-1} (^{o}C d)$^{-1}$)	5.8	8.2*	4.1	6.7*	7.6	7.5	1.4

Table 2. Activity (μmol m^{-2} s^{-1}) and activation state (%) of enzymes in leaves of Areces (A) and Gaitan (G) clovers and fescue developed at 25°C (HT), 12°C (LT) and transferred from 25 to 12°C (HLT). Determinations in parallel with the assays described in Fig. 3. Data are means of 3 replicates. LSD, least significant differences (P<0.05) for comparison with plants developed at 25°C. Significant differences are marked with an asterisk.

		Areces			Gaitan			Fescue			LSD	
		HT	HLT	LT	HT	HLT	LT	HT	HLT	LT	A & G	Fescue
Rubisco	Total	22	17	26*	20	18	30*	54	59	51	6.3	8.9
	Initial	13	10	16*	11	9.7	19*	27	40*	34*	4.3	6
	%	60	61	64	55	54	66	50	70*	68*	11.7	16.5
sFBPase	Total	16	9.5*	32*	13	14	42*	32	36	46*	3.5	4.3
	Initial	14	8.4*	23*	10	12	39*	13	18*	20*	2.1	2.6
	%	91	88	74*	77	87*	93*	40	50*	44	6.5	8.0
cFBPase		4.4	2.3*	4.1	3.3	2.6	5.1*	6.3	6	9.6*	1.0	1.4
SPS	Vmax	0.5	1.0*	2.7*	0.3	0.9*	3.2*	4.8	7.8*	21*	0.5	3.1
	Limiting	0.2	0.5*	1.6*	0.1	0.5*	2.1*	1.8	3.0*	12*	0.3	1.1
	%	38	52*	59*	26	59*	65*	38	34	65*	8.8	14.4

Legends to Figures

Figure 1. Effect of temperature on (A) dry weight, (B) green area and (C) dry weight, and (D) green area on a thermal time basis in Areces (squares) and Gaitan (triangles) clovers and fescue (circles). Plants were grown at 25°C (open symbols) or 12°C (closed symbols). Data are means of 3 replicates.

Figure. 2. Photosynthetic oxygen evolution at 900 μmol m^{-2} s^{-1} photon flux density, saturating CO_2 and at the growth temperature of youngest fully expanded leaves of Areces (A) and Gaitan (G) clovers and fescue (F) developed at 25°C (empty squares), 12°C (filled squares) and transferred from 25 to 12°C (hatched squares), measured 1 day after the shift to low temperature (25-28-day-old plants) in HT and HLT plants and at the same growth stage (41-44-day-old plants) in LT leaves. Data are means of 3 replicates. Vertical bars represent LSD (P<0.05) for temperature contrasts. Significant differences are marked with an asterisk.

Figure 3. Contents of (A) glucose, (B) fructose, (C) sucrose, (D) starch and (E) 'fructan' in leaves of Areces (A) and Gaitan (G) clovers and fescue (F) developed at 25°C (empty squares), 12°C (filled squares) and transferred from 25 to 12°C (hatched squares). 'Fructan' refers to the low molecular weight fructan contents as estimated from the fructose in excess of glucose after invertase hydrolysis. The levels of starch and fructan are in hexose equivalents. At the sixth to seventh-leaf stage (25–28-day-old HT and HLT plants and 41-44-day-old LT) the youngest fully-expanded leaf of 8 plants in each replicate was harvested, immediately transferred to liquid nitrogen under illumination and pooled. Data are means of 3 replicates. Vertical bars represent LSD (P<0.05) for

contrasts with plants developed at 25°C. Significant differences are marked with an asterisk.

Figure 4. Metabolite levels and ratios in leaves of Areces (A) and Gaitan (G) clovers and fescue (F) developed at 25°C (empty squares), 12°C (filled squares) and transferred from 25 to 12°C (hatched squares). Determinations in parallel with the assays described in Fig. 3. Data are means of 3 replicates. Vertical bars represent LSD (P<0.05) for contrasts with plants developed at 25°C. Significant differences are marked with an asterisk.

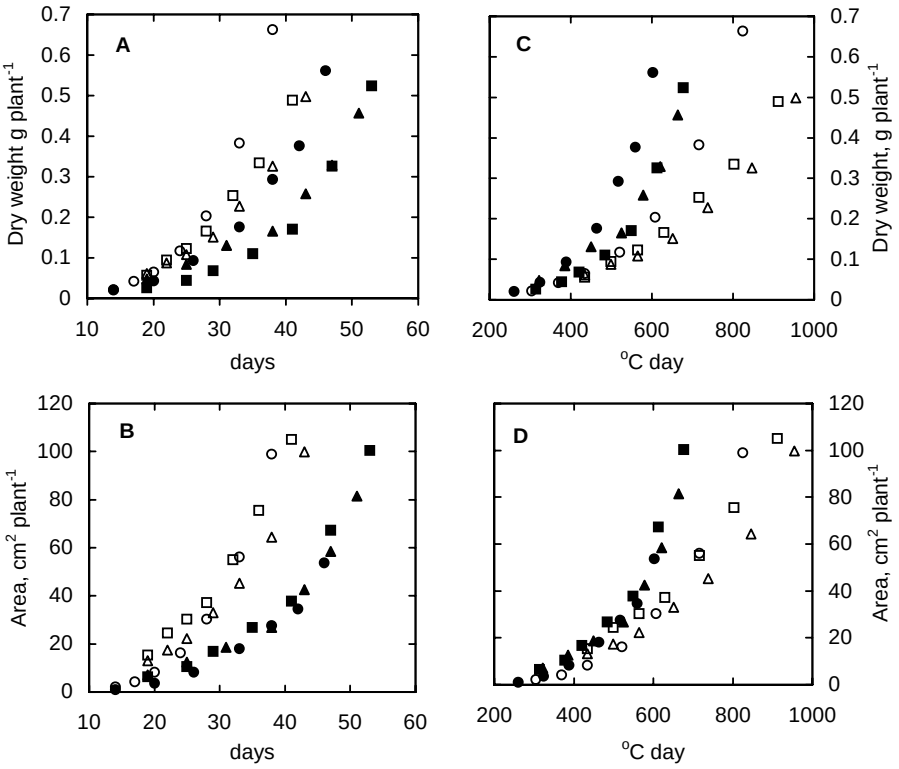
A
B
C
D
Dry weight g plant-1
Area, cm2 plant-1
Dry weight, g plant-1
days
°C day
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
120
100
80
60
40
20
10
30
50
200
400
600
800
1000

Figure 1.

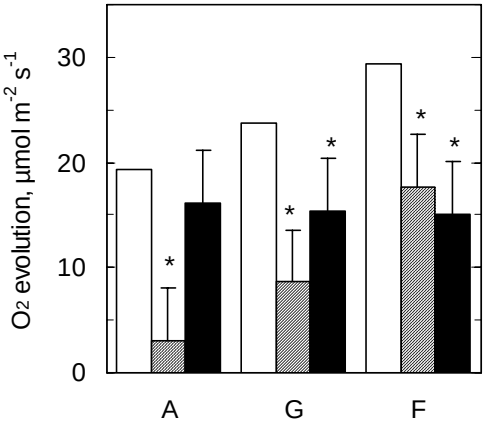

Figure 2.

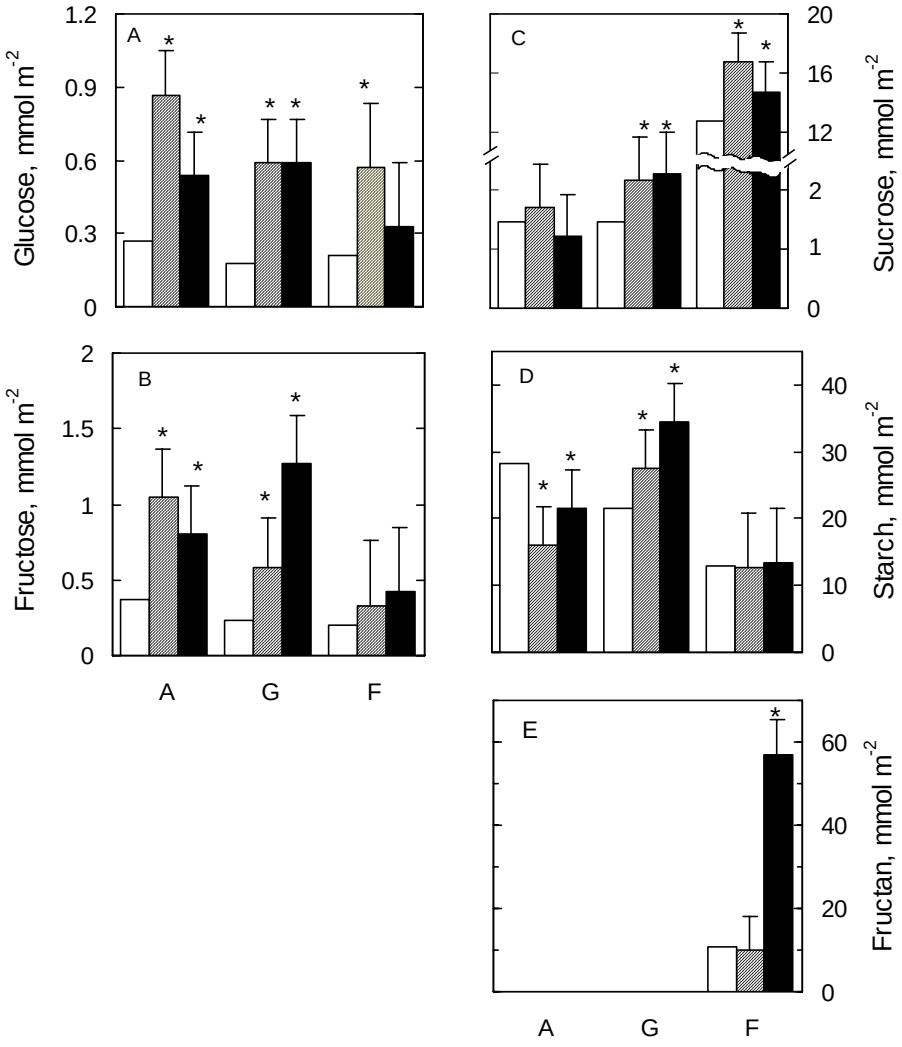
A
B
C
D
E
Glucose, mmol m-2
Fructose, mmol m-2
Sucrose, mmol m-2
Starch, mmol m-2
Fructan, mmol m-2
1.2
0.9
0.6
0.3
0
2
1.5
1
0.5
20
16
12
2
1
40
30
20
10
60
40
20
A
G
F

Figure 3.

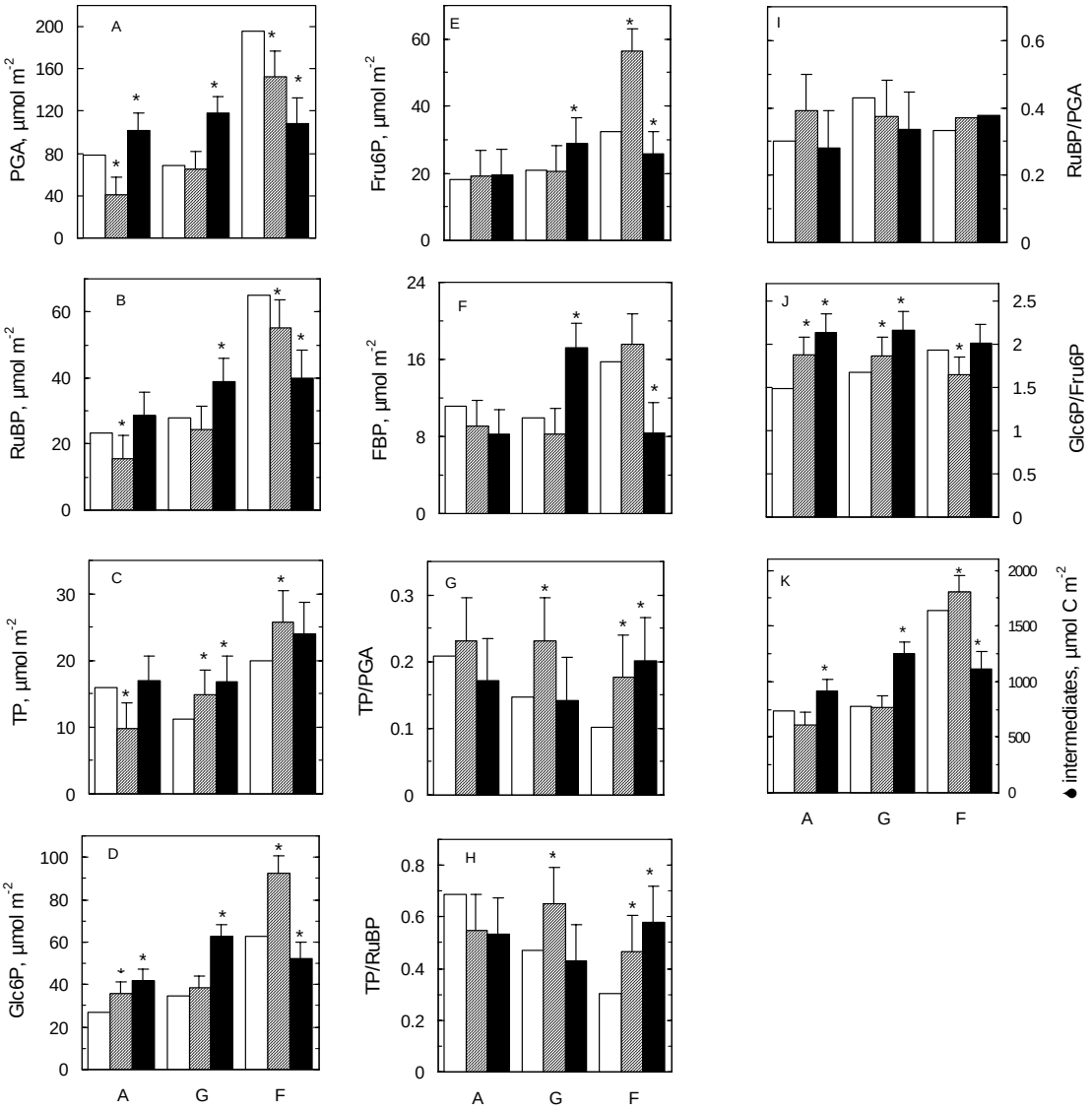
A
PGA, µmol m-2
E
Fru6P, µmol m-2
I
RuBP/PGA
B
RuBP, µmol m-2
F
FBP, µmol m-2
J
Glc6P/Fru6P
C
TP, µmol m-2
G
TP/PGA
K
intermediates, µmol C m-2
D
Glc6P, µmol m-2
H
TP/RuBP
A
G
F

Figure 4.